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Evaluation of Ligand-Dependent Changes in AR Structure Using Peptide Probes

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Mutations in the AR are frequently found in relapsed prostate cancers, some of which permit antiandrogens as well as nonandrogenic compounds to function as androgens. However, the molecular mechanism(s) by which these mutations enable this aberrant AR pharmacology is still unknown. To explore this issue, we used a series of LxxLL-containing peptides (L, leucine; x, any amino acid) to probe the conformation of the AF-2/coactivator binding pocket of AR and AR mutants when complexed with different ligands. We have identified in a previous study two peptides that bind to the wild-type AR in an agonist-dependent manner. Interestingly, we found these same peptides also interacted with several AR variants that are frequently found in antihormone refractory prostate cancers, in the presence of either androgens or antiandrogens. This suggests that the agonist activity of antiandrogens and other physiologically relevant ligands occurs because they, in the background of these mutations, allow AR-AF2 to adopt an active conformation. Initially, this result appeared to contradict the findings of others that suggest that coactivator binding to AR-AF2 is not required for AR activity. In probing this paradox further, however, we determined that the role of AR-AF2 appears to be to stabilize the overall structure of the receptor, allowing the amino terminus to interact with appropriate coactivators. This conclusion is supported by our finding that overexpression of the AF2-binding peptides blocks the interaction between the amino and carboxyl termini of AR but does not attenuate AR transcriptional activity. This can be explained by the fact that overexpression of the LxxLL-containing peptide or the amino terminus of AR appears to have a similar effect on the AR-ligand binding domain, as both have the ability to stabilize agonist binding by decreasing ligand off-rate. Thus, we believe that resistance in certain prostate cancers occurs as a consequence of receptor mutations that enable antagonist-and/or nonclassical ligand-bound AR to present a wild-type-like AF-2 conformation. (*Molecular Endocrinology* 16: 647–660, 2002)

THE AR belongs to the nuclear hormone receptor superfamily of ligand-activated transcription factors that modulate diverse biological functions in response to either endogenous or exogenous stimuli (1, 2). Members of this gene family share sequence homologies and exhibit similar modular domain structures (3–5). Nuclear receptors contain a carboxyl-terminal ligand-binding domain (LBD) that allows them to bind their cognate ligands, and a central DNA binding region (DBD) that permits their interaction with specific DNA sequences located within the promoter regions of the target genes that they regulate. In most receptors, the LBD also harbors a transactivation function domain (AF-2), a key protein-protein interaction module that allows these receptors to direct the assembly of multiprotein transcription complexes at target genes. Those receptors that bind with high affinity to steroid hormones such as androgens, estrogens, glucocorticoids, mineralcorticoids, and progestins, also contain at least one additional activation function domain (AF-1) at the amino terminus of the receptor. The two AF domains in these receptors can either synergize or function independently to enable the regulation of gene transcription in a cell-type and/or tissue-specific manner (6–8).

In the past few years, it has become clear that the nuclear receptors do not function alone and that the activities they manifest are the result of their ability to interact with other cellular coregulator proteins, coactivators, and corepressors (9–11). Structural and biochemical studies have determined that upon binding their cognate hormone, the AF-2 of the nuclear receptors undergoes an activating conformational change, enabling the receptor to interact with various coactivators (12, 13). Most of the validated coactivators contain a leucine-rich LxxLL-motif (L, leucine; and x, any amino acid) that mediates their interactions with the receptor AF-2 (14). Crystallographic studies using the ERα as a model revealed that when ER is activated by an agonist, the LxxLL-motif from the coactivator GRIP-1 (glucocorticoid receptor interacting protein-1) binds in a hydrophobic surface atop the ligand-binding pocket (12). It appears that most receptors have evolved to utilize this hydrophobic surface as a major docking site for coactivators, and not surprisingly mu-

Abbreviations: AF-1, Activation function-1; DBD, DNA binding region; DHT, 5α-dihydrotestosterone; GRIP-1, glucocorticoid receptor interacting protein-1; L, leucine; LBD, ligand binding domain; OHF, hydroxyflutamide; SRC-1, steroid receptor coactivator-1; x, any amino acid.

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tations of residues that line this hydrophobic pocket generally render the receptor transcriptionally inactive. Similarly, changing the conserved leucine residues in the LxxLL-motif into alanine in the known coactivators abrogates their ability to interact and potentiate receptor activity. Indeed, the LBD (containing the AF-2) of most receptors when tethered to DNA is able to induce reporter gene transcription in an agonist-dependent manner, for it in itself is capable of recruiting the LxxLL-containing coactivators. With that being said, some of these coactivators have been shown to interact with multiple sites on the receptor. SRC-1 (steroid receptor coactivator-1) and GRIP-1, for example, can interact with both the AF-1 and AF-2 of PR and ER using different regions of the protein (15–17). The physiological significance of the alternate contact points between receptor and coactivators remains to be determined.

In contrast to other nuclear receptors, the AR-LBD when expressed alone manifests minimal transcriptional activity. Although the full-length AR has been shown to interact also with SRC-1 and GRIP-1, unlike their interaction with other nuclear receptors, the LxxLL-motif in these coactivators appears not to be sufficient for these interactions (8, 18, 19). In addition, it has also been reported that the C terminus of the p160s can interact and potentiate the AR AF-1 (20, 21). It is believed therefore that AR may have unique coactivator binding characteristics. Various AR-interacting proteins have been identified by yeast two-hybrid screens using different AR fragments as bait, including ARA70, ARA54, ARA55, ARA24, ARA160, FHL-2, ARIP3, ARIP4, etc. (22–28). These proteins bind to different regions of AR and when overexpressed in cells potentiate AR activity. The physiological significance and the detailed mechanisms of action of these proteins, however, remains to be determined.

Protein-protein interactions govern almost all biological processes, including activation and repression by nuclear receptors. Not surprisingly, analysis of the ligand-induced structural alterations in the receptor, and how they influence its ability to interact with comodulators, has become one of the most important areas in the study of receptor pharmacology. Crystallographic studies of the ER-LBD suggest that the conformational changes which occur upon agonist binding permit the docking of the coactivators through their LxxLL motifs (12, 29, 30). Upon antagonist binding, on the other hand, receptor conformation is altered so that the helix-12 of the LBD is repositioned to occupy the coactivator-binding pocket, thereby blocking the access of coactivators. These studies provide a structural basis for the agonist and antagonist activities of receptor ligands. The crystal structure of the agonist-bound AR-LBD is very similar to that of the other nuclear receptors (31). Still unpublished is the structure of antagonist-bound AR; therefore, the conformational differences between agonist- and antagonist-activated receptor, and the impact of these structural alterations on receptor function, are not known. In addition, many antiandrogen-resistant prostate tumors contain one or more mutations in the AR-LBD, which allows the receptor to manifest an aberrant ligand-specificity. For instance, mutation of the codon 877 from threonine to alanine permits this mutant AR to activate gene transcription in the presence of hydroxyflutamide, an antagonist of the wild-type receptor (32, 33). The crystal structure of this mutant AR-LBD revealed that the replacement of threonine 877 with alanine enlarges the ligand binding pocket enabling the accommodation of the bulky side chains on the C17 of steroids (34). This may explain why this mutant receptor can bind to a variety of other hormones that normally do not interact with AR (31, 34, 35). Clearly, conformational changes within the AR-LBD have a significant impact on receptor pharmacology. Without a structure of the antagonist-bound receptor, however, it is still unclear how a compound can switch from being an antagonist to an agonist, as has been observed on the AR/T877A mutant.

In a previous study, we used a series of short peptides to probe ligand-induced conformational changes in ER (36–38). This peptide binding analysis complemented ongoing crystallographic studies and revealed that different ER ligands allow the presentation of different protein-protein interaction surfaces on ER and facilitate the interaction of the receptor with different coregulatory proteins. Most importantly, this work also led to the discovery of at least one mechanism to explain how antiestrogen-resistant tumors may develop in breast cancers, where the tumor cells switch from recognizing antiestrogens as antagonists to agonists. In this current study, we present data using a similar approach to the study of mechanisms underlying agonist/antagonist activities of AR-ligands, a first step in the development of new antiandrogens for the treatment of prostate cancer and other endocrinopathies.

RESULTS

Probing AR Conformation Using LxxLL-Containing Peptides

In a previous study, we used phage display to assess ligand-induced structural changes within the ER and identified several peptide probes whose binding to the receptor is conformation sensitive (36, 38, 39). Some of these peptides contain an LxxLL-motif and recognize agonist-activated ER but not that activated by antagonists. Because these peptides were found to be capable of interacting with several other nuclear receptors, we wished to determine if any of them could be used to recognize the active conformation of the AR. We found only two peptides, D11 and D30, that were capable of interacting with AR in the presence of the agonist 5α-dihydrotestosterone (DHT). In the current study, we characterized these peptides further

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and assessed the interaction of AR with each peptide in the presence of either the agonist DHT, or two different antagonists: hydroxyflutamide (OHF) and bicalutamide. A mammalian two-hybrid assay was performed to evaluate the interactions between AR and all of the LxxLL-containing peptides identified previously (38, 40). For this assay we expressed each peptide as a Gal4-DBD fusion and used a modified AR, VP16-AR, in which the acidic VP16 activation domain was cloned onto the extreme amino terminus of AR. Interactions between AR and the peptides were detected by measuring the expression of a luciferase reporter gene containing five copies of the Gal4-response element. The results of a representative analysis are shown in Fig. 1. While these ERα- and/or ERβ-interacting peptides were shown to interact with multiple other nuclear receptors (38, 40), we were able to identify only two peptides (D11 and D30) that could interact with agonist-activated AR (Table 1). Interestingly, AR did not interact with the LxxLL-motifs contained within SRC-1 and GRIP-1 in this assay, in agreement with previous findings by other investigators (8, 18, 19). This indicated to us that the protein-protein interaction surfaces presented on AR are unusual and that the receptor may regulate transcription using a distinct set of coactivators. Nevertheless, the D11 and D30 peptides specifically interacted with agonist- but not antagonist-activated AR, suggesting that these two peptides do indeed detect an active conformation of this receptor. Although the D11 and D30 peptides both contain an "SSRLxxLLM" motif, we do not know as yet which amino acids are required for AR-binding. Such an analysis has been confounded by the recent identification of another LxxLL peptide in our laboratory (LX23), which interacted

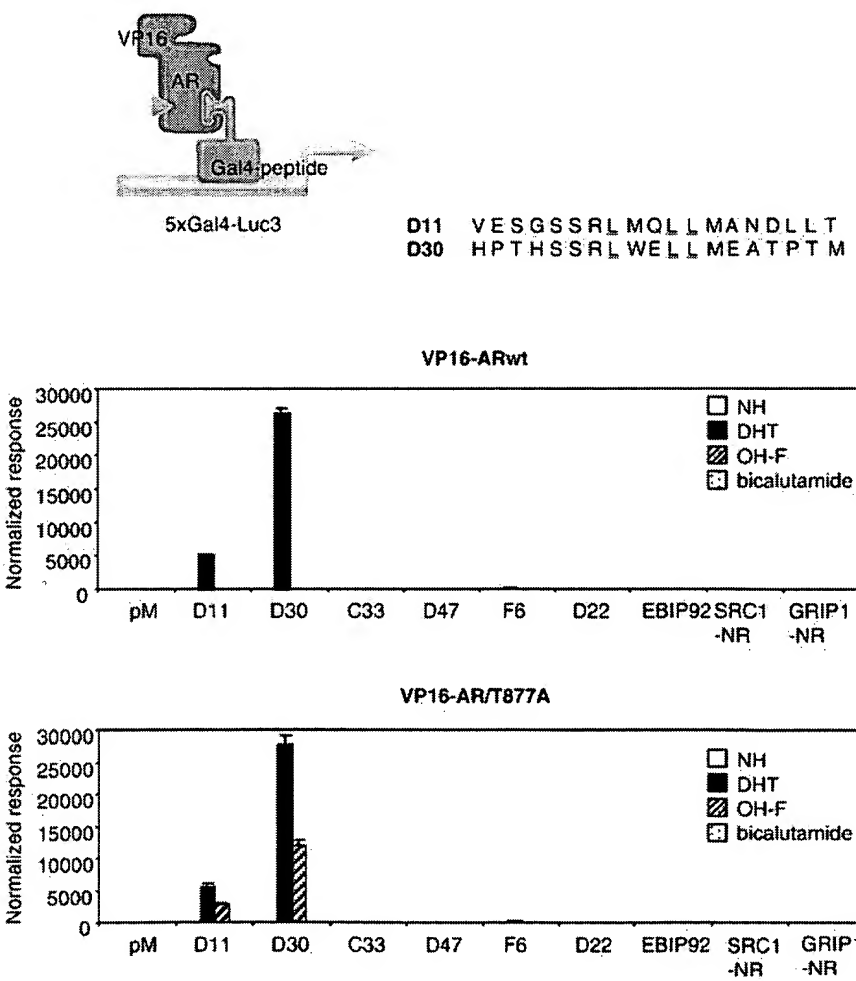

Fig. 1. The T877A Mutation Permits Antiandrogen-Bound AR to Adopt a Conformation Similar to that of the Wild-Type Receptor

Different LxxLL-motif containing peptides were fused to the Gal4-DBD, and the full-length ARs (wild-type and the T877A mutant) were modified to include a VP16-activation domain at their amino termini. Interactions between peptides and AR were determined by measuring the expression of a reporter gene containing five copies of the Gal4-response elements. CV-1 cells were transfected with different peptide-Gal4DBD constructs together with either the VP16-ARwt or the VP16-AR/T877A expression plasmid, and reporter constructs 5xGal4Luc3 and pCMV-βgal. After transfection, cells were treated with either vehicle control (NH), 100 nM DHT, 100 nM OHF, or 1 μM bicalutamide for 16 h. Luciferase activity was measured and normalized to the activity of the coexpressed β-galactosidase.

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Table 1. The AR Interacts with Only a Small Subset of LxxLL-Containing Peptides

Peptide	Sequence	AR Interaction
D11	VESGSSRLMQLLMANDLLT	+
D30	HPTHSSRLWELLMEATPTM	+
ER4	SSNHQSSRLIELLSR	–
D14	QEAHGPLLWNLLSRSDTDW	–
D47	HVYQHPLLLSLLSSEHESG	–
C33	HVEMHPLLMGLLMESQWGA	–
F6	GHEPLTLLERLLMDDKQAV	–
D22	LPYEGSLLLKLLRAPVEEV	–
D48	SQWENSILYSLLSDRVSLD	–
D43	AHGESSLLAWLLSGEYSSA	–
D40	SGWNESILYRLLQADAFDV	–
D15	PSGGSSVLEYLLTHDTSIL	–
F4	PVGEPGLLWRLLSAPVERE	–
RIP140/935-944	VLKQLLLSEN	–
C5	TVWERASLADLLEWQEEVR	–
293	SSIKDFPNLISLLSR	–
EBIP37	TGGGVSLLLHLLNTEQGES	–
EBIP41	RRDDFPLLISLLKDGALSQ	–
EBIP44	YGLKMSLLESLLREDISTV	–
EBIP45	MSYDMLSLYPLLTNSLLEV	–
EBIP51	FPAEFPLLTYLLERQGMDE	–
EBIP96	VESEFPYLLSLLGEVSPQP	–
EBIP49	VSSEGRLLIDLLVDGQQSE	–
EBIP53	DTPQSPLLWGLLSSDRVEG	–
EBIP60	GGTQDGYLWSLLTGMPEVS	–
EBIP66	SLPEEGFLMKLLTLEGDAE	–
EBIP70	VMGNNPILVSLLEEPSEEP	–
EBIP76	VLVEHPILGGLLSTRVDSS	–
EBIP87	QTPLLEQLLTEHIQQG	–
EBIP92	SVWPGPELLKLLSGTSVAE	–
EBIP56	GSWQDSLLLQLLNRTELMA	–
GRIP-1 NR1[a]	DSKGQTKLLQLLTTKSDQM	–
GRIP-1 NR2[a]	LKEKHKILHQLLQDSSSPV	–
GRIP-1 NR3[a]	KKKENALLRYLLDKDDTKD	–
SRC-1 NR1[a]	YSQTSHKLVKLLTTTAEQQ	–
SRC-1 NR2[a]	LTARHKILHRLLQEGSPSD	–
SRC-1 NR3[a]	ESKDHQLLRYLLDKDEKDL	–
FxxLF[b]	SKTYRGAFQNLFQSVREVIQNP	+

[a] The NR boxes from SRC-1 and GRIP-1 were not tested individually but as Gal4DBD fusion proteins containing all three NR boxes. The GRIP-1 construct contains amino acids 629–760 from GRIP-1 and the SRC-1 construct includes amino acids 621–765 from SRC-1.

[b] Amino acids 16–34 from the AR.

with AR but, outside of the LxxLL motif, does not resemble D11 or D30 (Kimbrel, E., and D. P. McDonnell, personal communication).

Several AR mutations have been identified in metastatic prostate cancer which, in addition to being activated by androgens, can also be activated by nonandrogenic compounds. For example, the AR/T877A mutant that contains an alanine at codon 877 instead of a threonine in the wild-type receptor, allows the antagonist OHF to function as an agonist (32, 33). This agonist/antagonist activity switch is considered to be one of the mechanisms by which some prostate tumors escape the inhibitory activity of antiandrogens. Similarly, resistance has been observed after treatment with the antiestrogen tamoxifen in breast cancer patients, where cancer cells switch from recognizing tamoxifen as an ER-antagonist to an agonist (41, 42). In a previous study, we discovered that although the conformation of ER induced by binding to tamoxifen prevents its interaction with p160 coactivators, it enables the presentation of surfaces on the receptor that may be used to recruit other novel coactivators (36, 39). We therefore proposed two scenarios that might explain the agonist/antagonist switch observed in prostate cancers that contains the T877A mutation. One possibility is that, analogous to the mechanism by which tamoxifen resistance arises in breast cancer, the T877A mutation may allow the OHF-bound AR to present a unique surface that permits its interaction with a coactivator with which it would not normally couple. The second possibility is that, in the background of the T877A mutation, OHF-bound AR adopts

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a conformation similar to that of the agonist-activated wild-type receptor. To distinguish between these two possibilities, we extended our use of peptide probes to an analysis of the conformation of the AR/T877A mutant in the presence of both DHT and OHF. Surprisingly, we found that OHF-activated AR/T877A interacted with the same peptides as agonist-bound AR (Fig. 1B). This result indicated to us that the agonist/antagonist switch in AR/T877A could be explained by the fact that, in the background of this mutation, the OHF-occupied mutant AR adopts a conformation similar to that of agonist-activated wild-type receptor. Importantly, neither the wild-type nor the T877A mutant is recruited to the D11 and D30 peptides in the presence of bicalutamide, a compound that has been shown previously to function as a pure antagonist for both ARs. These results indicate that the D11 and D30 peptides serve as sensitive probes that detect a receptor conformation that is compatible with transcriptional activation.

D11 and D30 Peptides Interact with the AR-LBD

Previously, we found that all the LxxLL peptides used in this study, including D11 and D30, bind to the coactivator docking surface within the ER-LBD (38). There is, however, evidence suggesting that the LxxLL motifs present in coactivators SRC-1 and GRIP-1 are not important for the docking of these coactivators with AR (18, 43). In addition, our two-hybrid assay also showed that most LxxLL motifs, including those from GRIP-1 and SRC-1, do not interact with AR. We therefore wished to determine if the D11 and D30 peptides function like other coactivator peptides and bind to the region within AR analogous to the coactivator binding pocket in other receptors. Using different fragments of AR in a mammalian two-hybrid assay, we determined that the DBD/LBD (AR507–919) fragment alone was sufficient for D30 (and D11, data not shown) binding (Fig. 2, B and C). In addition, truncation of the amino terminus did not influence peptide binding specificity because peptides that failed to bind the full-length AR

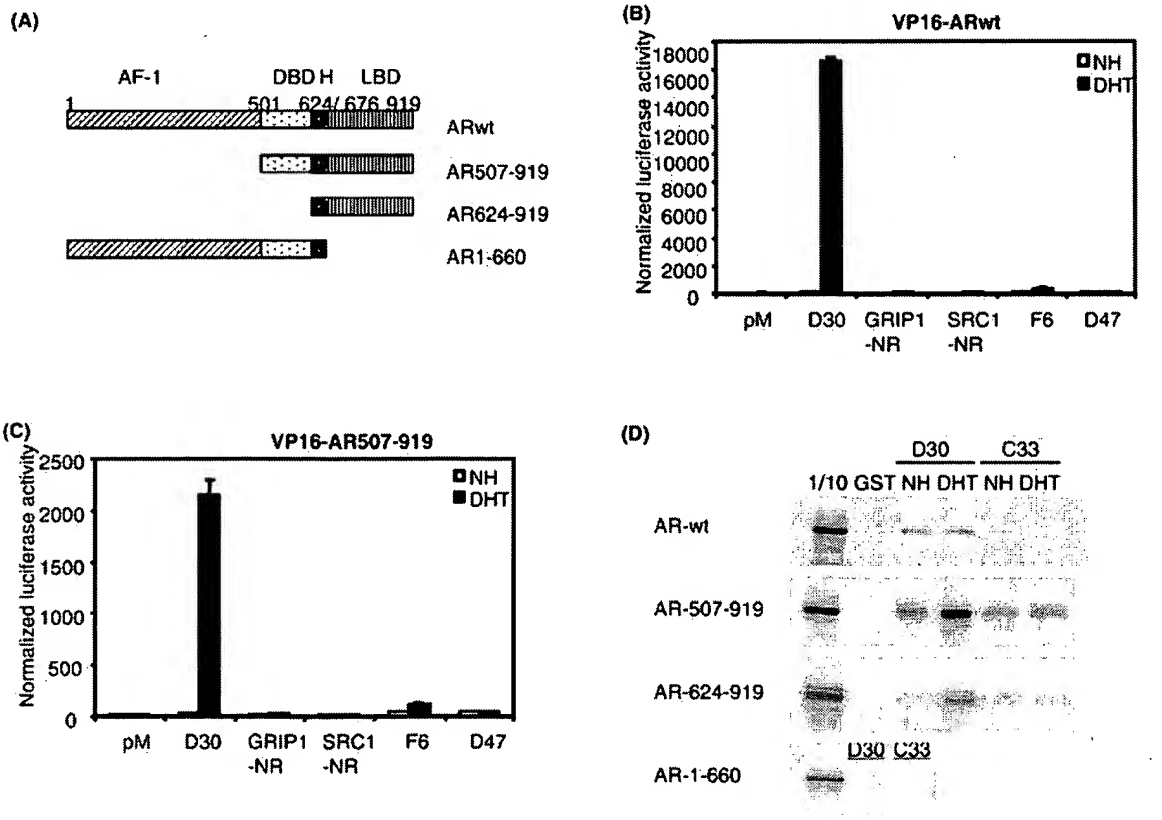

Fig. 2. The D30 Peptide Interacts with the LBD of AR

A schematic diagram of the different AR fragments used is shown in A. CV-1 cells were transfected with different Gal4DBD-peptide fusion constructs and either the VP16-ARwt (B), or the VP16-AR507–919 (C), expression plasmids, together with the 5xGal4Luc3 and pCMV-βgal reporters. After transfection, cells were treated with vehicle control or 100 nM DHT for 16 h and the luciferase and β-galactosidase activities were determined. D, Different fragments of AR were *in vitro* translated and labeled with ^{35}S-methionine. Bacterially expressed GST, GST-D30, and GST-C33 proteins were purified using glutathione-Sepharose beads, and equal amounts of these proteins were incubated with different *in vitro* translated AR fragments in the presence or absence of 1 μM DHT. Nonspecific binding was reduced by four washes with PBST. Proteins remaining bound to the beads were resolved by SDS-PAGE and detected by autoradiography. 1/10, One tenth of the total input protein.

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were also unable to bind to the AR DBD-LBD. No interaction with the amino terminus (AR 1–660) was detectable in this assay (data not shown). Similar results were obtained in a GST-pull-down assay. Using the D30 peptide fused to the GST protein, we confirmed that all three constructs, the full length-AR, AR-DBD/LBD, and AR-LBD alone (AR624–919), but not the amino terminus of the receptor (AR1–660), interacted with the D30 peptide (Fig. 2D). Based on these data, we believe that the D30 peptide binds within the LBD of AR.

The D30 Binding Surface Does Not Overlap with the AR: Coactivator Interaction Surface

Because the binding site of the LxxLL peptides overlaps the coactivator docking surface on most of the nuclear receptors, it is not surprising that these peptides, when overexpressed in cells, competitively block coactivator recruitment and inhibit receptor transcriptional activity (38, 40). Because the D30 peptide interacts with AR in an agonist-dependent manner, we wished to determine if this peptide also binds at a site that coincides with or overlaps the coactivator-binding surface on AR. In transfected CV-1 cells, therefore, we overexpressed a two-copy peptide construct, pM-2xD30, to see if it could disrupt AR transcriptional activity. A two-copy peptide was used because we had previously determined that it is more efficient than a single-copy peptide in disrupting ER transcriptional activity (38). Because the D30 peptide also interacts with ER, we were not surprised to see that 2xD30 efficiently inhibited ER transcriptional activity when overexpressed in these cells. We were surprised, however, to find that the 2xD30 only marginally inhibited AR activity when assayed on the MMTV-Luc reporter gene (Fig. 3). Overexpression of this peptide had very little, if any, effect on AR activity when different reporter constructs were used. Similar results were obtained using other cell lines (data not shown). We considered three possible explanations for these results. First, the surface on AR where D30 interacts is not easily accessible when the receptor is DNA bound. Secondly, the D30 binding site on AR does not coincide with a coactivator binding surface; therefore, coactivator binding is not affected. Thirdly, it is possible that the D30 peptide may not have high enough affinity to compete with coactivators for binding to AR.

To investigate these possibilities, we first tested whether the conformation of the DNA-bound AR can still be recognized by the D30 peptide. A modified mammalian two-hybrid assay was used in which the expressed AR was allowed to interact with a reporter gene containing an AR response element and the D30 peptide was made so as to contain a VP16 fusion at its amino terminus. Recruitment of the VP16-D30 fusion protein to AR would result in an increase of transcription from the reporter gene. We confirmed with this assay that the D30 peptide is capable of interacting with the DNA-bound AR (data not shown). This result suggests to us that either the D30 binding site on AR does not coincide with a coactivator-binding surface, therefore, coactivator binding is not affected, or that the D30 peptide may not have high enough affinity to compete with coactivators for binding to AR.

The D30 Peptide and the Amino Terminus of AR Bind at Overlapping Surfaces on AR-LBD

The findings of several studies from other investigators indicate that the amino and carboxyl termini of AR can interact (7, 18, 44). He *et al.* (45) have also shown recently that an LxxLL-like motif, FxxLF, located at the amino terminus of AR mediates this interaction. Consequently, we considered that the D11 and D30 peptides might be binding to the same surface as this FxxLF motif and therefore used a reconstituted AR transcription assay to address this possibility. In CV-1 cells, the AR DBD-LBD (AR507–919) alone displayed minimal transcriptional activity even when DHT was added to the medium, confirming that the DBD-LBD itself does not recruit coactivators efficiently (Fig. 4A). Because the amino terminus of AR is able to interact

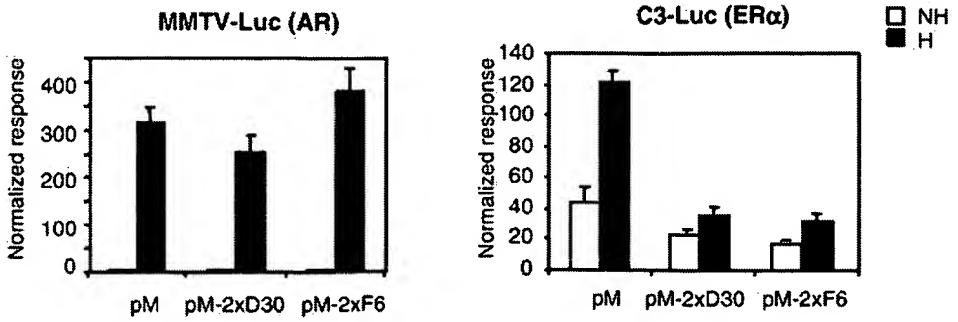

Fig. 3. The D30 Binding Surface on AR Does Not Overlap with that Required for Coactivator Binding

CV-1 cells were transfected with (A) MMTV-Luc, pCMVβgal, and RS-AR or (B) C3-Luc, pCMVβgal, and RST7-ERα in the presence of either pM, pM-2xD30 or pM-2xF6 as indicated. After transfection, cells were treated with either vehicle control or (A) 100 nM DHT or (B) 100 nM E2 for 16 h. Luciferase activity was measured and normalized to the activity of the coexpressed β-galactosidase.

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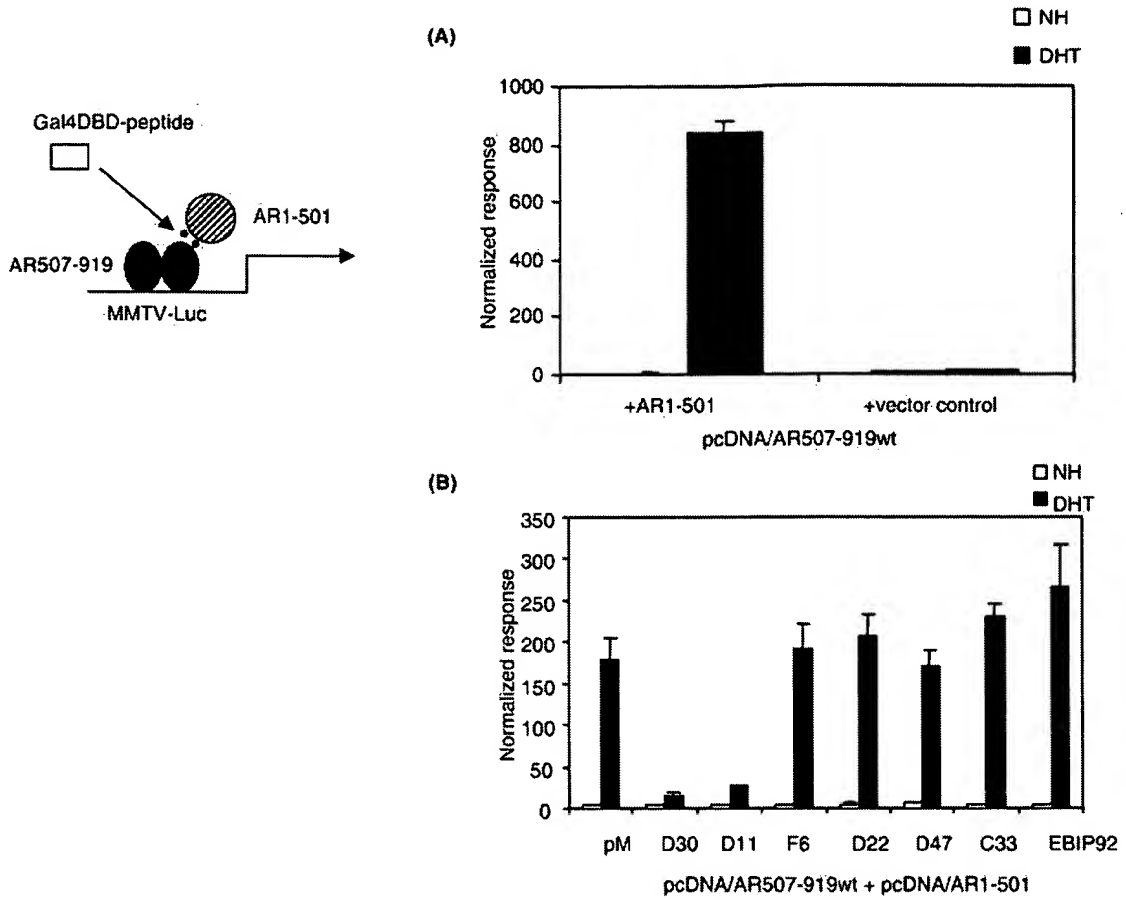

Fig. 4. The D11 and D30 Peptides Bind at a Site in the AR-LBD that Overlaps or Coincides with the Site Where the NH2 Terminus Binds

A, The AR-DBD/LBD alone has minimal transcriptional activity; coexpression of the AR1–501 (N terminus) restores ligand-dependent reporter gene activation. CV-1 cells were transfected with MMTV-Luc, pCMV-βgal, and the AR-DBD/LBD expression plasmid pcDNA/AR507–919wt, together with a control vector or a vector expressing AR1–501. After transfection, cells were treated with vehicle control or 100 nM DHT for 16 h and then assayed for luciferase and β-galactosidase activities. B, The D30 and D11 peptides efficiently blocked the interaction between the amino terminus of AR and the AR-LBD. CV-1 cells were transfected with pcDNA/AR507–919wt, pcDNA/AR1–501, MMTV-Luc, and pCMV-βgal, together with plasmids expressing different Gal4DBD-peptide fusions. Cells were induced and assayed as in A.

with the AR-LBD and is capable of recruiting coactivators on its own, coexpression with the DBD-LBD fragment AR1–501 restores DHT-induced reporter gene activity (Fig. 4A). To test if the D11 and D30 peptides bind to the same surface on AR-LBD as the AR amino terminus, we overexpressed these peptides in the reconstituted system, and found that D11 and D30, but not other LxxLL-containing peptides, could interrupt the AR N/C termini interaction (Fig. 4B). We conclude, therefore, that the D11 and D30 peptides are binding to either the same or overlapping surfaces on LBD where the amino terminus of the receptor binds.

It has been shown in the past that the interaction of the amino terminus of AR with the LBD could stabilize ligand binding to the receptor (46). Because the D30 peptide appears to be binding at the same or overlapping site where the amino terminus of the receptor also binds, we wished to determine if the D30 peptide has the same effect of retaining the ligand in the ligand-binding pocket. This was accomplished by measuring the dissociation rate of a nonmetabolizable ligand, R1881, from AR (Fig. 5). The dissociation half-time (T1/2) of R1881 from full-length AR was approximately 124 min in our assay, and the T1/2 was shortened to about 21 min when the AR507–919 was analyzed in a similar manner. Coexpression of the amino terminus of AR (AR1–501) prolonged the T1/2 to about 47 min and coexpression of the 2xD30 had a similar effect. This result further confirms our theory that the D30 peptide is binding to the same surface on AR-LBD, where the amino terminus of AR also binds. In addition, it suggests that the D30 may serve the same purpose as the amino terminus of the receptor, that of stabilizing the ligand in its binding pocket. This result may also explain why overexpression of the D30 peptide did not inhibit AR transcriptional activity because it functions similarly to the AR N terminus in

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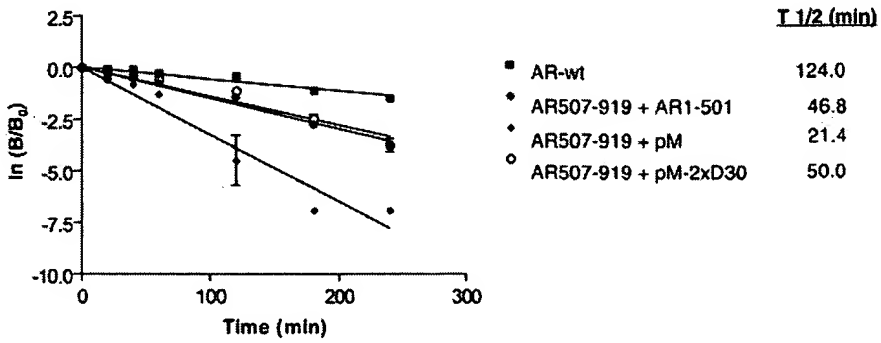

Fig. 5. The D30 Peptide Functions Similarly to the Amino Terminus of AR in Stabilizing Ligand Binding in the AR-LBD

CV-1 cells were transfected with expression plasmids for either 1) wild-type AR alone; 2) AR507–919 plus AR1–501; 3) AR507–919 plus Gal4DBD (pM); or 4) AR507–919 plus Gal4DBD-2xD30 (pM-2xD30). Twenty-four hours after transfection, cells were labeled with 5 nM of ^{3}H-R1881 for 2 h and then a 10,000-fold excess of cold R1881 was added at different time points. Cells were washed four times with PBS to remove nonspecific binding and then lysed for scintillation counting and protein concentration measurement.

stabilizing ligand binding. Although we cannot totally rule out the possibility that the D30 peptide may not have high enough affinity to compete with coactivators for binding to AR, our data is most consistent with the hypothesis that the D30/AR interaction resembled more of the AR N/C termini interaction than of cofactor:AR interaction.

Formation of a D30 Docking Site on AR Is Required for Agonist Activity

Although the D30 binding surface within AR is not a coactivator docking site, we were intrigued by the fact that the D30 peptide appeared to recognize the transcriptionally competent AR conformation. We wondered if formation of a D30 binding pocket on AR is required for it to manifest agonist activity and whether the D30:AR interaction indeed predicts AR transcriptional activity. To probe this hypothesis, we compared several agonists and antagonists of AR for their ability to induce reporter gene activation as well as the interaction of AR with D30. The result of this assay is shown in Fig. 6. With MMTV-Luc as a reporter, only DHT was a full agonist for both wild-type AR and the AR/T877A mutant. OHF and RU486 had very little activity when wild-type AR was cotransfected; in contrast, OHF efficiently induced MMTV-Luc expression in the presence of the T877A mutation (Fig. 6A). The ability of these compounds to activate reporter gene expression was compared in parallel with their ability to facilitate the D30:AR interaction (Fig. 6B). The full agonist DHT induced more than a 500-fold interaction between AR and the D30 peptide. RU486, although not as efficient as DHT, induced a 15- to 20-fold interaction between D30 and wild-type AR as well as the T877A AR, which parallels the weak agonist activity of this compound on both ARs. On the other hand, OHF, a potent activator of the T877A mutant but not the wild-type AR, also induced a robust interaction between D30 and VP16-ART877A, but not the wild-type VP16-AR.

Receptor activation, the process of converting AR from an inactive to a transcriptionally active form, requires nuclear translocation, receptor dimerization, DNA binding and recruitment of cofactor proteins. To assess which step requires the formation of a D30 binding pocket, we first tested whether DNA binding can be achieved without the formation of this pocket. We fused both wild-type and T877A AR to VP16 to determine if the ability of compounds to deliver AR to DNA correlates with their ability to induce a D30 binding pocket on AR. This analysis revealed that the wild-type AR activated by OHF was not delivered to DNA efficiently (Fig. 6C). In the presence of the T877A mutation, however, OHF-bound receptor was brought to the DNA as efficiently as that activated by DHT. Interestingly, while RU486 could efficiently deliver both AR-wt and AR/T877A to DNA, the RU486-bound AR was not recruited to the D30 peptide efficiently. This result suggests that the D30 binding pocket is not required for AR to translocate, dimerize, and bind to DNA, but rather is required for events downstream of DNA binding. We have also tested a number of other AR agonists and antagonists (47), and the results confirm that the ability of a compound to induce a D30 binding surface on AR correlates very well with transcriptional activation.

Because we have been able to show that the D30 peptide and the amino terminus of AR compete for binding to the same site on the AR-LBD, we tested whether the AR N-/C-interaction also tracks with transcriptional activation. We found that the AR amino terminus (AR1–501) could be recruited to the AR-DBD/LBD fragment (AR507–919) only when the LBD is in an active conformation, the same conformation that permits the D30 peptide to bind (Fig. 6D). Similar results were also obtained using Gal4DBD-fusions containing the first FxxLF motif in the AR NH2-terminal domain (^{23}FQNLF^{27}) (data not shown). This result suggests that although the AR-LBD is not directly involved in recruiting coactivators, it contributes to shaping a con-

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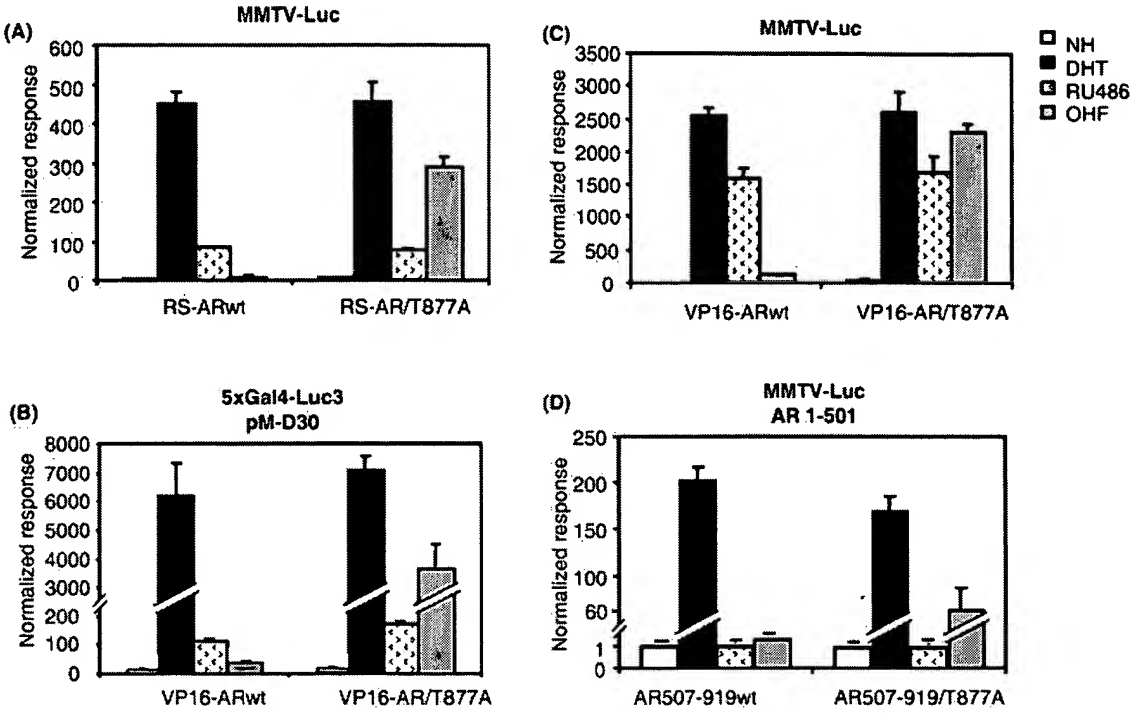

Fig. 6. The Formation of a D30-Binding Pocket on AR Is Required for Its Transcriptional Activity

A reporter gene assay was used to analyze the agonist or antagonist activity of AR ligands. CV-1 cells were transfected with either wild-type or T877A mutant AR expression plasmids, RS-ARwt and RS-AR/T877A, respectively; MMTV-Luc and pCMV-βgal were used as reporter constructs in this assay. B, The mammalian two-hybrid assay was performed to determine the ability of ligand-AR complexes to recruit the D30 peptide. CV-1 cells were transfected with 5xGal4Luc3, pCMV-βgal and pM-30, together with either VP16-ARwt or VP16-AR/T877A. C, The ARwt and AR/T877A were expressed as fusion proteins to the VP16-acidic activation domain to bypass the need for AR-specific coactivators required for gene transcription, allowing the assessment of the ability of ligands to deliver receptors to DNA. CV-1 cells were transfected with pVP16-ARwt or pVP16-AR/T877A together with MMTV-Luc and pCMV-βgal. D, The ability of ligand-AR/LBD complexes to recruit the amino terminus of AR was analyzed. CV-1 cells were transfected with MMTV-Luc, pCMV-βgal and pcDNA-AR1–501, together with either pcDNA-AR507–919wt or pcDNA-AR507–919/T877A. After transfection, cells were treated with either vehicle alone (NH), 100 nM DHT, 100 nM OHF, or 100 nM RU486 as indicated for 16 h before the luciferase and β-galactosidase activities were determined.

formation that is required for AR transcriptional activity. Interestingly, the N/C interaction did not predict the partial agonist activity of RU486 because no N/C interaction was observed in the presence of RU486 (Fig. 6D). This result indicates that the D30/AR interaction may be a more sensitive and/or accurate predictor of AR transcriptional activation.

Several missense mutations have been identified in prostate cancers that appear to increase the agonist efficacy of some androgenic and nonandrogenic ligands. Therefore, we next evaluated whether mutations in the LBD, outside the AF-2 domain, would behave like the T877A mutant and permit the receptor to adopt an active conformation in the presence of a wide variety of ligands. The majority of AR mutations found in prostate cancers are located between codons 670–678, 701–730 (signature sequence), and 874–910 (48). Based on homology modeling, the residues 668–671 do not contribute directly to ligand binding and are positioned away from the ligand binding pocket (49), which suggests that mutations in this region are less likely to influence the binding of the D30 peptide. We therefore decided to focus our analysis on AR mutations found in the latter two regions. The L701H mutant has a reduced sensitivity to the natural ligand DHT; however, it can be activated by physiological concentrations of cortisol (50). As shown in Fig. 7, A and B, the D30 peptide indeed interacted less efficiently with the DHT-activated L701H receptor but gains the ability to interact with this AR variant in the presence of 10 nM cortisol. Another mutation in the signature sequence region, V715M, was shown to have an increased response to progesterone, androsterone, and a number of other endogenous hormones (51, 52). In our analysis, we found that the AR-V715M also recruited the D30 peptide more efficiently in the presence of these ligands (Fig. 7, C and D). In addition to the T877A mutation, another AR variant, H874Y, also contained within the region flanking AF2 (codons 874–910), was found to recognize E2 and hydroxyflutamide as AR agonists (53, 54). In our analysis, we found that the same D30-binding pocket was also formed on the surface of this mutant AR in the presence of these ligands. A full dose-response curve of various ligands used in these analyses (10^{-12}–10^{-6} M) was also performed to ensure that the

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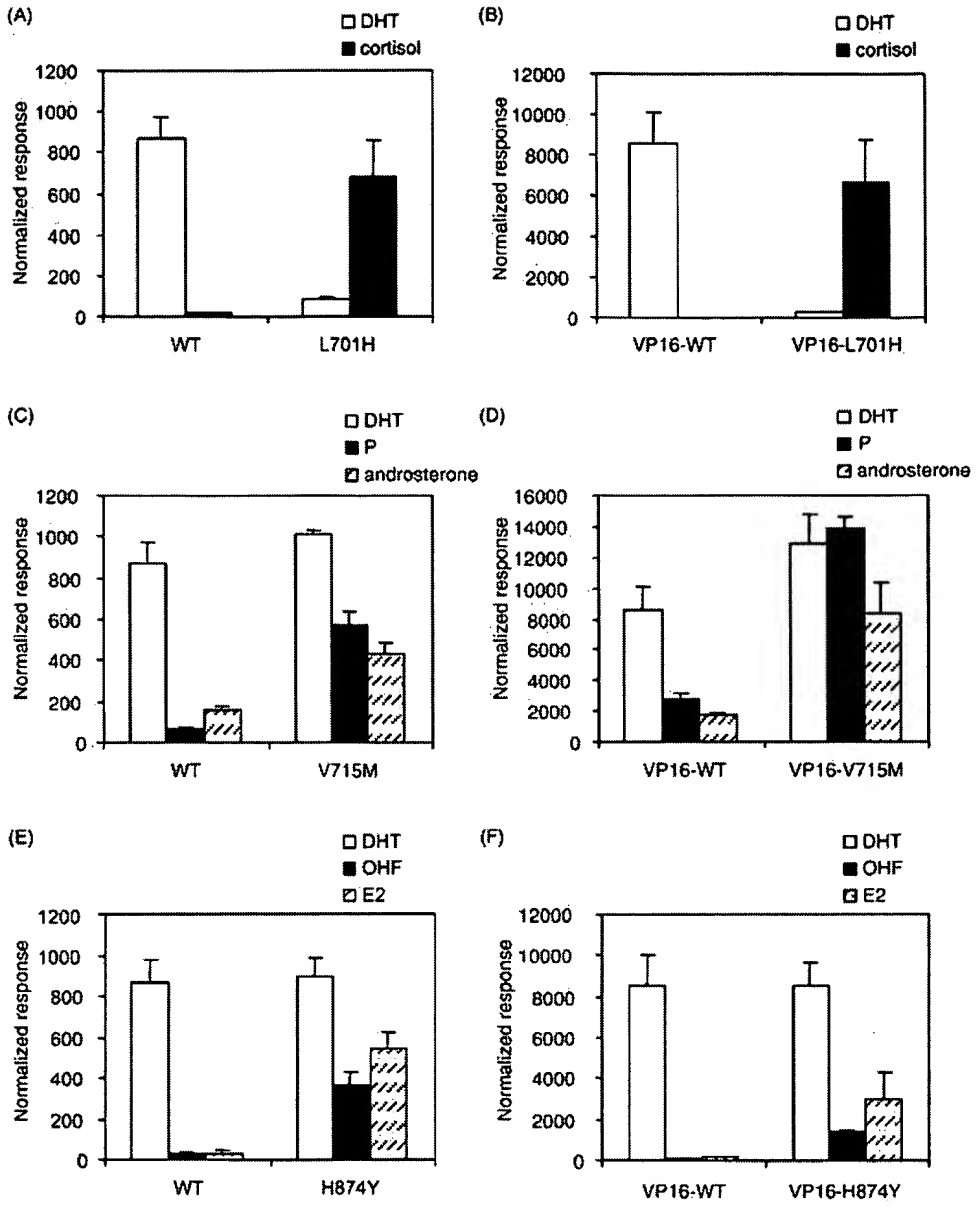

Fig. 7. The D30-Peptide Predicts the Transcriptionally Active Conformation of Various Mutant ARs

A, C, and E, CV-1 cells were transfected with either wild-type or mutant AR expression plasmids, MMTV-Luc and pCMV-βgal. B, D, and F, CV-1 cells were transfected with 5xGal4Luc3, pCMV-βgal and pM-30, together with either VP16-ARwt or VP16-AR mutants. After transfection, cells were treated with either 0.1 nM DHT, 100 nM OHF, 1 nM progesterone (P), 1 nM 17β-estradiol (E2), 10 nM cortisol, or 100 nM androsterone as indicated for 16 h before the luciferase and β-galactosidase activities were determined.

observed phenotype of the cloned mutants reflected that which has been published previously (data not shown). We concluded, therefore, that regardless of the class of mutations, that gain of function phenotype of AR-LBD variants tracks with the acquisition of the ability of AR to adopt an active AF-2 conformation.

DISCUSSION

In this study, we analyzed the ligand-induced structural changes in AR using peptide probes, and found two peptides, D11 and D30, which detect a conformation of AR that is compatible with transcriptional activation. Upon binding an agonist, the LBD of most nuclear receptors adopts an active conformation which allows the presentation of a coactivator binding pocket, permitting the docking of coactivator proteins via a helical LxxLL motif (12–14). We have demonstrated in this report that in the presence of an activating ligand, the AR-LBD undergoes a similar conformational change, allowing the docking of an LxxLL motif contained within the D11 and D30 peptides. While we and others have shown previously that dif-

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ferent receptors have distinct LxxLL motif preferences (38, 55), it is noteworthy that AR appears to be one of the most selective, for it interacts with only a very limited subset of LxxLL sequences. Furthermore, although the docking site for these LxxLL peptides in other nuclear receptors is used to recruit coactivators, the analogous region in AR appears to have a different function. Our data agree with previous findings by other investigators that the AR-LBD utilizes this surface to couple with its own amino terminus (44, 45). This interaction slows down the dissociation of ligand from the LBD, maintains the receptor in an active conformation, and thus may allow a more efficient recruitment of coactivators and the subsequent target gene transcription.

Langley *et al.* (44) have shown previously that the OHF binding induced conformational changes within the T877A mutant but not the wild-type AR-LBD, which permitted its interaction with the NH2 terminus of the receptor and that correlated with the activation of this mutant receptor by OHF (44). He *et al.* (45) have also identified an LxxLL-like motif, FxxLF, located within the amino terminus of AR that appears to be responsible for docking the amino terminus of AR to the LBD. Consistent with these data, our results using combinatorial peptide approach have reached the same conclusion and reinforced the hypothesis that the interaction between the amino and carboxyl termini of AR may be required for its transcriptional activity. Our peptide probes appear to have higher sensitivity when compared with the other assays since the partial agonist activity of RU486 is only detected by the D30 peptide but not the N-/C-interaction assays (Fig. 6D). In addition to the ligands examined in this study, we have also tested a large number of other compounds (Ref. 47 and data not shown). All of the results obtained confirm that AR transcriptional activity can be accurately predicted by assaying either the AR N/C termini interaction or the recruitment of VP16-AR to the D30 peptide and that the peptide approach is always the more sensitive of the two assays.

Several factors that appear to contribute to the development of antiestrogen resistance in breast cancers have been identified, including amplification of coactivators, down-regulation of corepressors, and ectopic interactions of ER with cofactor proteins (39, 56–58). We found that the T877A mutation allows AR to adopt an active conformation regardless of whether it is bound by an agonist or antagonist. Because this same active conformation is found in the agonist-bound wild-type AR, it suggests that the consequence of this amino acid change may be to allow mutant AR to appear as a wild-type receptor in target cells. Similarly, the L701H, V715M, and H874Y AR mutants also appeared to function in the same manner by allowing these AR variants to function like wild-type AR in the presence of nonclassical AR ligands. It has been observed that AR mutations occur at a higher frequency in advanced prostate cancers compared with early stage tumors (59–61). All current hormonal manipulations focus on controlling the access of androgens to their cellular receptor, either through elimination of androgens in the circulation or by using an antagonist to compete for their binding to AR. In view of the high frequency of AR mutations identified in advanced cancers, we think that these manipulations will all fail ultimately. A more promising approach would be to develop drugs that target different sites on AR. Based on our theory that the AR amino and carboxyl termini interaction is required for its transcriptional activity, we believe that a peptide or a small molecule that can target the disruption of this interaction would be useful as a therapy for the treatment of antiandrogen refractory prostate tumors. In addition, if the consequence of all AR mutations is to allow the presentation of the same active conformation, it would suggest that both the mutant and wild-type AR may use the same surface to recruit coactivators. Consequently, targeting the interaction between AR and the coactivator(s) required for its transcriptional activity has the potential of being an effective therapy for the treatment of metastatic prostate cancers.

MATERIALS AND METHODS

Reagents and Plasmids

The RS-AR and VP16-AR were gifts from K. Marschke (Ligand Pharmaceuticals, Inc., San Diego, CA) and the plasmids expressing the AR mutants were generated using the QuickChange site directed mutagenesis kit (Stratagene, La Jolla, CA). The 5xGal4Luc3, MMTV-Luc, and pM-peptide plasmids were described previously (38, 62). VP16-AR507–919, VP16-AR624–919, VP16-AR1–660, pcDNA-ARwt, pcDNA-AR507–919, pcDNA-AR624–919, pcDNA-AR1–660, pcDNA-AR1–501 and pM-AR624–919 were generated using PCR amplified AR fragments, and were subcloned into pVP16, pcDNA3 and pM vectors (CLONTECH Laboratories, Inc., Palo Alto, CA, and Invitrogen, Carlsbad, CA). The pVP16–2xD30 and pM-2xD30 plasmids were made in a similar fashion as described before (38), in which two copies of the D30 peptide were fused to the VP16 acidic activation domain and Gal4-DBD, respectively. The GST-D30 plasmid was made by subcloning the D30 peptide into the pGex-6p1 vector (Amersham Pharmacia Biotech, Piscataway, NJ). All the cell culture media and supplements were purchased from Life Technologies, Inc. (Grand Island, NY). CV-1 cells were obtained from ATCC (Manassas, VA). R1881 and ^{3}H-R1881 were obtained from NEN Life Science Products (Boston, MA). DHT was purchased from Sigma (St. Louis, MO). Hydroxyflutamide and bicalutamide were gifts from K. Gaido (CIIT, Research Triangle Park, NC) and Nobex, Inc. (Research Triangle Park, NC).

Cell Culture and Transfection

Monkey kidney CV-1 cells were grown in minimum essential medium plus 8% fetal bovine serum, essential amino acids and sodium pyruvate. Lipofectin-mediated transfection was performed essentially as described (38). Hormones were added after cells recovered from transfection and then all were incubated for 16 h before assaying. Luciferase and β-galactosidase activities were determined as described (38).

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GST Pull-Down

Different fragments of AR were *in vitro* translated using pcDNA-ARwt, pcDNA-AR507–919, pcDNA-AR624–919, and pcDNA-AR1–660 as templates and the TNT-rabbit reticulocyte lysate system (Promega Corp., Madison, WI) in the presence of ^{35}S-methionine (Amersham Pharmacia Biotech). GST and GST-D30 proteins were expressed in *Escherichia coli* BL21 cells and purified using glutathione-Sepharose (Amersham Pharmacia Biotech). Sepharose beads containing equal amounts of GST or GST-D30 protein were incubated with *in vitro* translated AR fragments, in the presence or absence of 1 μM DHT overnight at 4 C. The beads were washed 4 times with PBST (PBS containing 0.1% Triton X-100), and proteins remaining bound to the beads were analyzed by SDS-PAGE and visualized with autoradiography.

Measurement of Dissociation Rates

CV-1 cells were seeded in 24-well plates and transfected overnight with different combinations of plasmids as indicated in the figure legend. Transfections were stopped by replacing the transfection mix with phenol-red free medium containing 8% charcoal-stripped FBS, and incubating overnight. For dissociation rate measurement, cells were labeled with 5 nM ^{3}H-R1881 for 2 h, and 10,000-fold excess of cold R1881 was added at different time points. Nonspecific binding was determined from cells that were treated with 5 nM ^{3}H-R1881 in the presence of 10,000-fold excess of cold R1881. The reactions were stopped by washing cells three times with PBS. Cells were dissolved in 200 μl of 1× SDS-buffer (1% SDS; 10 mM Tris, pH 6.8; 10% glycerol), incubated for 10 min at room temperature, and then 300 μl of 10 mM Tris (pH 8.0) was added and the contents were mixed thoroughly by repeat pipetting. For scintillation counting, 400 μl of sample was mixed with 3.5 ml CytoScint (ICN Biochemicals, Inc., Costa Mesa, CA) and counted for 1 min in the Beckman LS-6000 sc scintillation counter (Beckman Coulter, Inc., Fullerton, CA). Protein concentrations were determined by BCA assay (Pierce Chemical Co., Rockford, IL). The specific binding was normalized to the amount of total protein in the sample. The data was plotted and T1/2 calculated using the software Prism 3 (GraphPad Software, Inc., San Diego, CA).

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REFERENCES

1. Chang C, Kokontis J, Liao S 1988 Molecular cloning of human and rat complementary DNA encoding androgen receptors. Science 240:324–326
2. Lubahn DB, Joseph DR, Sullivan PM, Willard HF, French FS, Wilson EM 1988 Cloning of human androgen receptor complementary DNA and localization to the X chromosome. Science 240:327–330
3. Evans RM 1988 The steroid and thyroid hormone receptor superfamily. Science 240:889–895
4. Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P 1995 The nuclear receptor superfamily: the second decade. Cell 83:835–839
5. Beato M, Herrlich P, Schutz G 1995 Steroid hormone receptors: Many actors in search of a plot. Cell 83: 851–857
6. Tzukerman MT, Esty A, Santiso-Mere D, Danielian P, Parker MG, Stein RB, Pike JW, McDonnell DP 1994 Human estrogen receptor transactivational capacity is determined by both cellular and promoter context and mediated by two functionally distinct intramolecular regions. Mol Endocrinol 8:21–30
7. Doesburg P, Kuil CW, Berrevoets CA, Steketee K, Faber PW, Mulder E, Brinkmann AO, Trapman J 1997 Functional *in vivo* interaction between the amino-terminal, transactivation domain and the ligand binding domain of the androgen receptor. Biochemistry 36:1052–1064
8. Berrevoets CA, Doesburg P, Steketee K, Trapman J, Brinkmann AO 1998 Functional interactions of the AF-2 activation domain core region of the human androgen receptor with the amino-terminal domain and with the transcriptional coactivator TIF2 (transcriptional intermediary factor 2). Mol Endocrinol 12:1172–1183
9. Glass CK, Rose DW, Rosenfeld MG 1997 Nuclear receptor coactivators. Curr Opin Cell Biol 9:222–232
10. Jenster G 1998 Coactivators and corepressors as mediators of nuclear receptor function: an update. Mol Cell Endocrinol 143:1–7
11. Robyr D, Wolffe AP, Wahli W 2000 Nuclear hormone receptor coregulators in action: diversity for shared tasks. Mol Endocrinol 14:329–347
12. Shiau AK, Barstad D, Loria PM, Cheng L, Kushner PJ, Agard DA, Greene GL 1998 The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. Cell 95:927–937
13. Nolte RT, Wisely GB, Westin S, Cobb JE, Lambert MH, Kurokawa R, Rosenfeld MG, Willson TM, Glass CK, Milburn MV 1998 Ligand binding and co-activator assembly of the peroxisome proliferator-activated receptor-γ. Nature 395:137–143
14. Heery DM, Kalkhoven E, Hoare S, Parker MG 1997 A signature motif in transcriptional co-activators mediates binding to nuclear receptors. Nature 387:733–736
15. McInerney EM, Tsai MJ, O'Malley BW, Katzenellenbogen BS 1996 Analysis of estrogen receptor transcriptional enhancement by a nuclear hormone receptor coactivator. Proc Natl Acad Sci USA 93:10069–10073
16. Webb P, Nguyen P, Shinsako J, Anderson C, Feng W, Nguyen MP, Chen D, Huang SM, Subramanian S, McKinerney E, Katzenellenbogen BS, Stallcup MR, Kushner PJ 1998 Estrogen receptor activation function 1 works by binding p160 coactivator proteins. Mol Endocrinol 12:1605–1618
17. Onate SA, Boonyaratanakornkit V, Spencer TE, Tsai SY, Tsai MJ, Edwards DP, O'Malley BW 1998 The steroid receptor coactivator-1 contains multiple receptor interacting and activation domains that cooperatively enhance the activation function 1 (AF1) and AF2 domains of steroid receptors. J Biol Chem 273:12101–12108

EXHIBIT D

18. He B, Kemppainen JA, Voegel JJ, Gronemeyer H, Wilson EM 1999 Activation function 2 in the human androgen receptor ligand binding domain mediates interdomain communication with the NH(2)-terminal domain. J Biol Chem 274:37219–37225
19. Hong H, Darimont BD, Ma H, Yang L, Yamamoto KR, Stallcup MR 1999 An additional region of coactivator GRIP1 required for interaction with the hormone-binding domains of a subset of nuclear receptors. J Biol Chem 274:3496–3502
20. Ma H, Hong H, Huang SM, Irvine RA, Webb P, Kushner PJ, Coetzee GA, Stallcup MR 1999 Multiple signal input and output domains of the 160-kilodalton nuclear receptor coactivator proteins. Mol Cell Biol 19:6164–6173
21. Irvine RA, Ma H, Yu MC, Ross RK, Stallcup MR, Coetzee GA 2000 Inhibition of p160-mediated coactivation with increasing androgen receptor polyglutamine length. Hum Mol Genet 9:267–274
22. Yeh S, Chang C 1996 Cloning and characterization of a specific coactivator, ARA70, for the androgen receptor in human prostate cells. Proc Natl Acad Sci USA 93: 5517–5521
23. Kotaja N, Aittomaki S, Silvennoinen O, Palvimo JJ, Janne OA 2000 ARIP3 (androgen receptor-interacting protein 3) and other PIAS (protein inhibitor of activated STAT) proteins differ in their ability to modulate steroid receptor-dependent transcriptional activation. Mol Endocrinol 14: 1986–2000
24. Kang HY, Yeh S, Fujimoto N, Chang C 1999 Cloning and characterization of human prostate coactivator ARA54, a novel protein that associates with the androgen receptor. J Biol Chem 274:8570–8576
25. Hsiao PW, Lin DL, Nakao R, Chang C 1999 The linkage of Kennedy's neuron disease to ARA24, the first identified androgen receptor polyglutamine region-associated coactivator. J Biol Chem 274:20229–20234
26. Fujimoto N, Yeh S, Kang HY, Inui S, Chang HC, Mizokami A, Chang C 1999 Cloning and characterization of androgen receptor coactivator, ARA55, in human prostate. J Biol Chem 274:8316–8321
27. Hsiao PW, Chang C 1999 Isolation and characterization of ARA160 as the first androgen receptor N-terminal-associated coactivator in human prostate cells. J Biol Chem 274:22373–22379
28. Muller JM, Isele U, Metzger E, Muller JM, Isele U, Metzger E 2000 FHL2, a novel tissue-specific coactivator of the androgen receptor. EMBO J 19:359–369
29. Brzozowski AM, Pike AC, Dauter Z, Hubbard RE, Bonn T, Engstrom O, Ohman L, Greene GL, Gustafsson JA, Carlquist M 1997 Molecular basis of agonism and antagonism in the oestrogen receptor. Nature 389:753–758
30. Pike AC, Brzozowski AM, Hubbard RE, Bonn T, Thorsell AG, Engstrom O, Ljunggren J, Gustafsson JA, Carlquist M 1999 Structure of the ligand-binding domain of oestrogen receptor β in the presence of a partial agonist and a full antagonist. EMBO J 18:4608–4618
31. Matias PM, Donner P, Coelho R, Thomaz M, Peixoto C, Macedo S, Otto N, Joschko S, Scholz P, Wegg A, Basler S, Schafer M, Egner U, Carrondo MA 2000 Structural evidence for ligand specificity in the binding domain of the human androgen receptor. Implications for pathogenic gene mutations. J Biol Chem 275:26164–26171
32. Wilding G, Chen M, Gelmann EP 1989 Aberrant response *in vitro* of hormone-responsive prostate cancer cells to antiandrogens. Prostate 14:103–115
33. Veldscholte J, Berrevoets CA, Ris-Stalpers C,Kuiper GG, Jenster G, Trapman J, Brinkmann AO, Mulder E 1992 The androgen receptor in LNCap cells contains a mutation in the ligand binding domain which affects steroid binding characteristics and response to antiandrogens. J Steroid Biochem Mol Biol 41:3–8
34. Sack JS, Kish KF, Wang C, Attar RM, Kiefer SE, An Y, Wu GY, Scheffler JE, Salvati ME, Krystek SR Jr, Weinmann R, Einspahr HM 2001 Crystallographic structures of the ligand-binding domains of the androgen receptor and its T877A mutant complexed with the natural agonist dihydrotestosterone. Proc Natl Acad Sci USA 98:4904–4909
35. Poujol N, Wurtz JM, Tahiri B, Lumbroso S, Nicolas JC, Moras D, Sultan C 2000 Specific recognition of androgens by their nuclear receptor. A structure-function study. J Biol Chem 275:24022–24031
36. Paige LA, Christensen DJ, Gron H, Norris JD, Gottlin EB, Padilla KM, Chang CY, Ballas LM, Hamilton PT, McDonnell DP, Fowlkes DM 1999 Estrogen receptor modulators each induce distinict conformational changes in ERα and ERβ. Proc Natl Acad Sci USA 96:3999–4004
37. McDonnell DP, Chang CY, Norris JD 2000 Development of peptide antagonists that target estrogen receptor-cofactor interactions. J Steroid Biochem Mol Biol 74: 327–335
38. Chang C, Norris JD, Gron H, Paige LA, Hamilton PT, Kenan DJ, Fowlkes D, McDonnell DP 1999 Dissection of the LXXLL nuclear receptor-coactivator interaction motif using combinatorial peptide libraries: discovery of peptide antagonists of estrogen receptors α and β. Mol Cell Biol 19:8226–8239
39. Norris JD, Paige LA, Christensen DJ, Chang CY, Huacani MR, Fan D, Hamilton PT, Fowlkes DM, McDonnell DP 1999 Peptide antagonists of the human estrogen receptor. Science 285:744–746
40. Hall JM, Chang CY, McDonnell DP 2000 Development of peptide antagonists that target estrogen receptor β-coactivator interactions. Mol Endocrinol 14:2010–2023
41. Tonetti DA, Jordan VC 1995 Possible mechanisms in the emergence of tamoxifen-resistant breast cancer. Anti-Cancer Drugs 6:498–507
42. Jordan VC 2000 How is tamoxifen's action subverted? J Natl Cancer Inst 92:92–94
43. Bevan CL, Hoare S, Claessens F, Heery DM, Parker MG 1999 The AF1 and AF2 domains of the androgen receptor interact with distinct regions of SRC1. Mol Cell Biol 19:8383–8392
44. Langley E, Zhou ZX, Wilson EM 1995 Evidence for an anti-parallel orientation of the ligand-activated human androgen receptor dimer. J Biol Chem 270:29983–29990
45. He B, Kemppainen JA, Wilson EM 2000 FXXLF and WXXLF sequences mediate the NH2-terminal interaction with the ligand binding domain of the androgen receptor. J Biol Chem 275:22986–22994
46. Langley E, Kemppainen JA, Wilson EM 1998 Intermolecular NH2-/carboxyl-terminal interactions in androgen receptor dimerization revealed by mutations that cause androgen insensitivity. J Biol Chem 273:92–101
47. Chang C-Y, Walther PJ, McDonnell DP 2001 Glucocorticoids manifest androgenic activity in a cell line derived from a metastatic prostate cancer. Cancer Res 61: 8712–8717
48. Buchanan G, Greenberg NM, Scher HI, Harris JM, Marshall VR, Tilley WD 2001 Collocation of androgen receptor gene mutations in prostate cancer. Clin Cancer Res 7:1273–1281
49. Buchanan G, Yang M, Harris JM, Nahm HS, Han G, Moore N, Bentel JM, Matusik RJ, Horsfall DJ, Marshall VR, Greenberg NM, Tilley WD 2001 Mutations at the boundary of the hinge and ligand binding domain of the androgen receptor confer increased transactivation function. Mol Endocrinol 15:46–56
50. Zhao XY, Malloy PJ, Krishnan AV, Swami S, Navone NM, Peehl DM, Feldman D 2000 Glucocorticoids can promote androgen-independent growth of prostate cancer cells through a mutated androgen receptor. Nat Med 6:703–706
51. Culig Z, Hobisch A, Cronauer MV,Cato AC, Hittmair A, Radmayr C, Eberle J, Bartsch G, Klocker H 1993 Mutant androgen receptor detected in an advanced-stage pros-

EXHIBIT D

tatic carcinoma is activated by adrenal androgens and progesterone. Mol Endocrinol 7:1541–1550
52. Peterziel H, Culig Z, Stober J, Hobisch A, Radmayr C, Bartsch G, Klocker H, Cato AC1995 Mutant androgen receptors in prostatic tumors distinguish between amino-acid-sequence requirements for transactivation and ligand binding. Int J Cancer 63:544–550
53. Tan J, Sharief Y, Hamil KG, Gregory CW, Zang DY, Sar M, Gumerlock PH, deVere White RW, Pretlow TG, Harris SE, Wilson EM, Mohler JL, French FS 1997 Dehydroepiandrosterone activates mutant androgen receptors expressed in the androgen-dependent human prostate cancer xenograft CWR22 and LNCaP cells. Mol Endocrinol 11:450–459
54. Taplin ME, Bubley GJ, Shuster TD, Frantz ME, Spooner AE, Ogata GK, Keer HN, Balk SP 1995 Mutation of the androgen-receptor gene in metastatic androgen-independent prostate cancer. N Engl J Med 332:1393–1398
55. McInerney EM, Rose DW, Flynn SE, Westin S, Mullen TM, Krones A, Inostroza J, Torchia J, Nolte RT, Assa-Munt N, Milburn MV, Glass CK, Rosenfeld MG 1998 Determinants of coactivator LXXLL motif specificity in nuclear receptor transcriptional activation. Genes Dev 12:3357–3368
56. Anzick SL, Kononen J, Walker RL, Azorsa DO, Tanner MM, Guan XY, Sauter G, Kallioniemi OP, Trent JM, Meltzer PS 1997 AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer. Science 277:965–968
57. Smith CL, Nawaz Z, O'Malley BW 1997 Coactivator and corepressor regulation of the agonist/antagonist activity of the mixed antiestrogen, 4-hydroxytamoxifen. Mol Endocrinol 11:657–666
58. Jackson TA, Richer JK, Bain DL, Takimoto GS, Tung L, Horwitz KB 1997 The partial agonist activity of antagonist-occupied steroid receptors is controlled by a novel hinge domain-binding coactivator L7/SPA and the corepressors N-CoR or SMRT. Mol Endocrinol 11:693–705
59. Suzuki H, Akakura K, Komiya A, Aida S, Akimoto S, Shimazaki J 1996 Codon 877 mutation in the androgen receptor gene in advanced prostate cancer: relation to antiandrogen withdrawal syndrome. Prostate 29: 153–158
60. Taplin ME, Bubley GJ, Ko YJ, Small EJ, Upton M, Rajeshkumar B, Balk SP 1999 Selection for androgen receptor mutations in prostate cancers treated with androgen antagonist. Cancer Res 59:2511–2515
61. Marcelli M, Ittmann M, Mariani S, Sutherland R, Nigam R, Murthy L, Zhao Y, DiConcini D, Puxeddu E, Esen A, Eastham J, Weigel NL, Lamb DJ 2000 Androgen receptor mutations in prostate cancer. Cancer Res 60:944–949
62. Wagner BL, Pollio G, Giangrande P, Webster JC, Breslin M, Mais DE, Cook CE, Vedeckis WV, Cidlowski JA, McDonnell DP 1999 The novel progesterone receptor antagonists RTI 3021–012 and RTI 3021–022 exhibit complex glucocorticoid receptor antagonist activities: implications for the development of dissociated antiprogestins. Endocrinology 140:1449–1458